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Supplemental Information

Spatial Ecology of the Human Tongue Dorsum Microbiome

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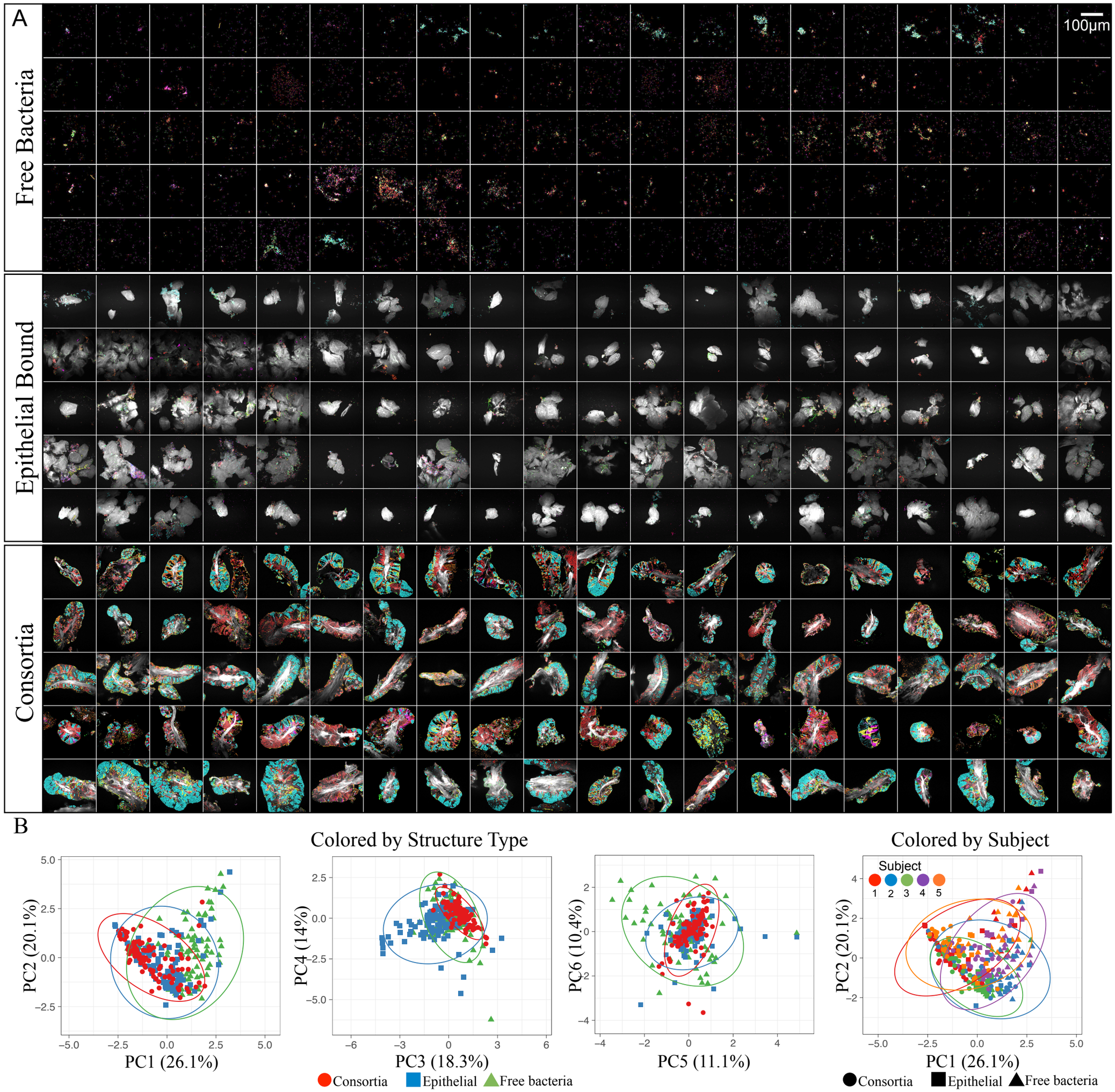
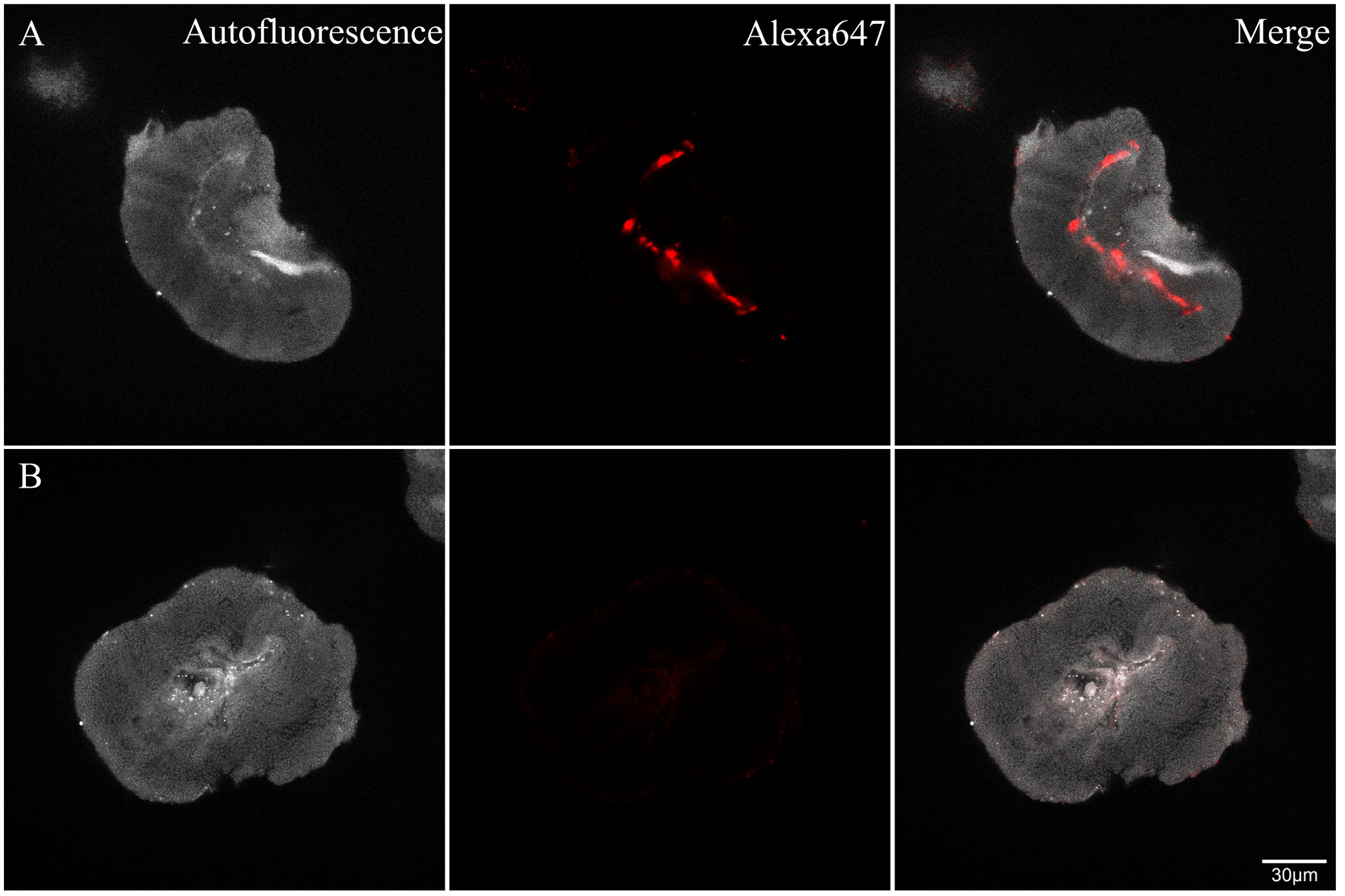
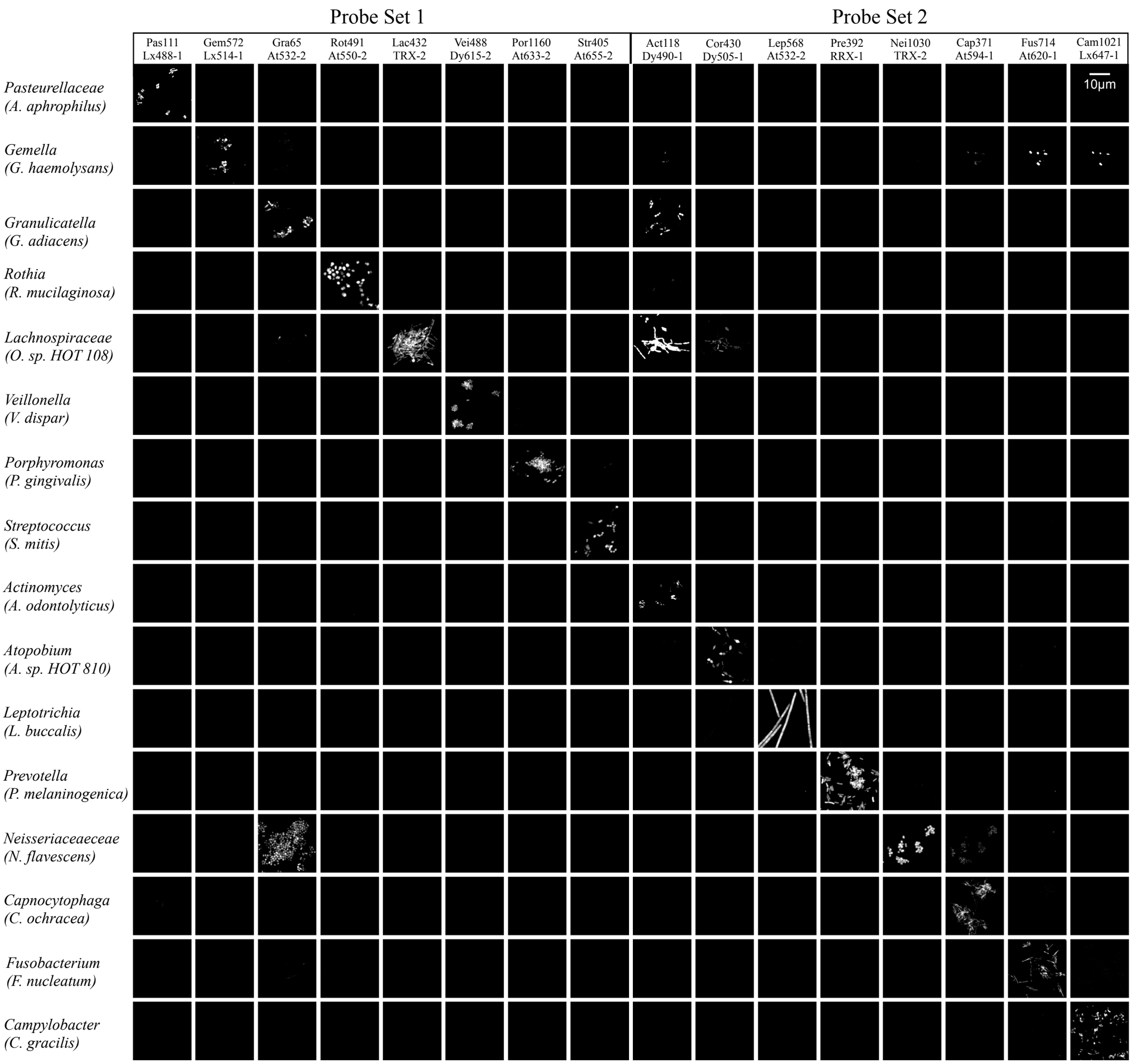


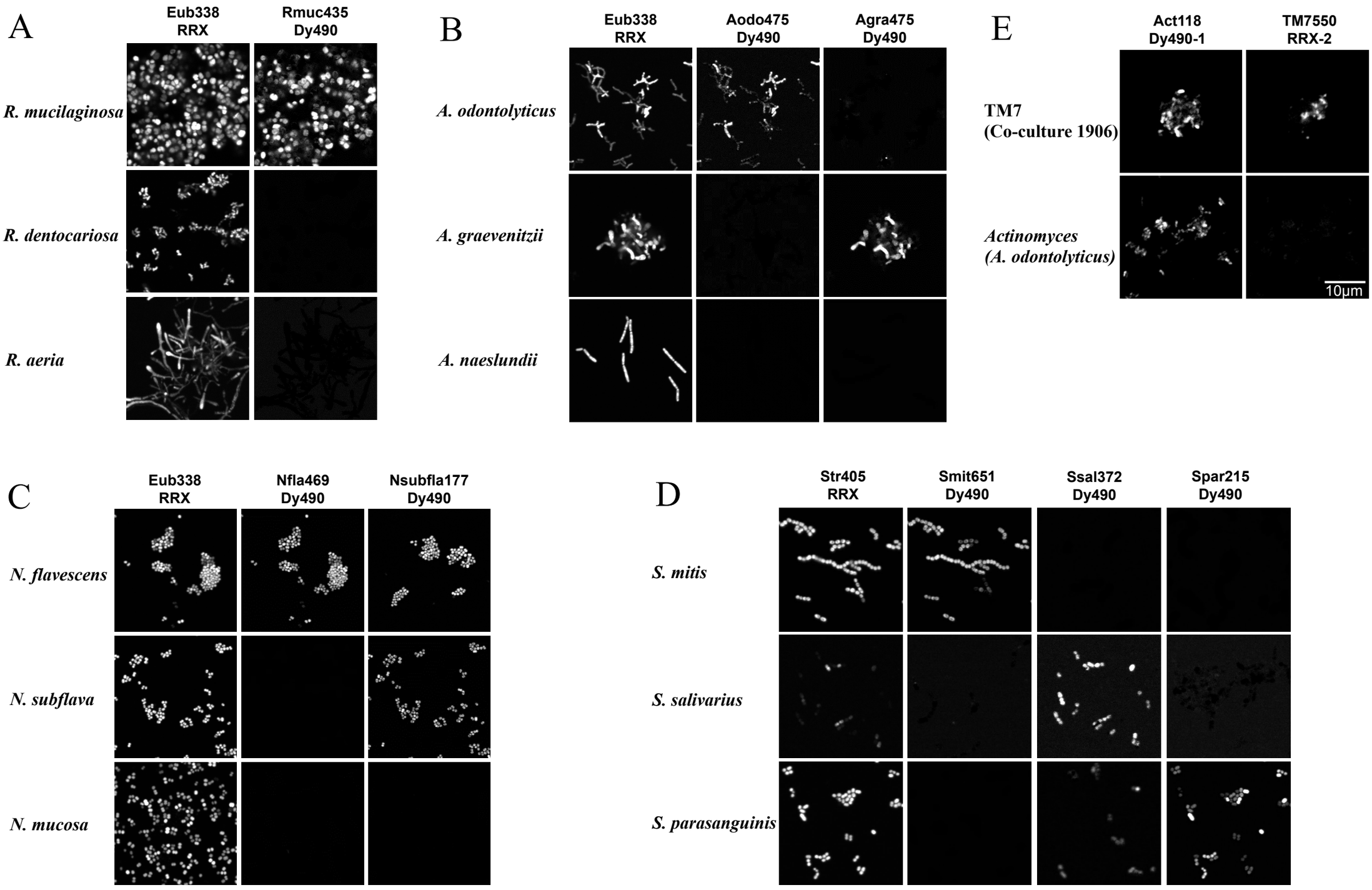
Supplemental Figure 1. **Montage of all 300 fields of view analyzed in Figure 2. Related to Figure 2.** Displayed are 300 images used for analysis of microbial composition and structure in 5 subjects. For each section of the figure (Free Bacteria, Epithelial Bound, and Consortia), each row represents a different donor subject and 20 fields of view from that subject are shown. To obtain the bar graphs in Figure 2, taxon channels were segmented to enumerate individual cells and counts were normalized to total counts for each field of view. Images display the segmented channels and corresponding autofluorescence channel for Epithelial Bound and Consortia.



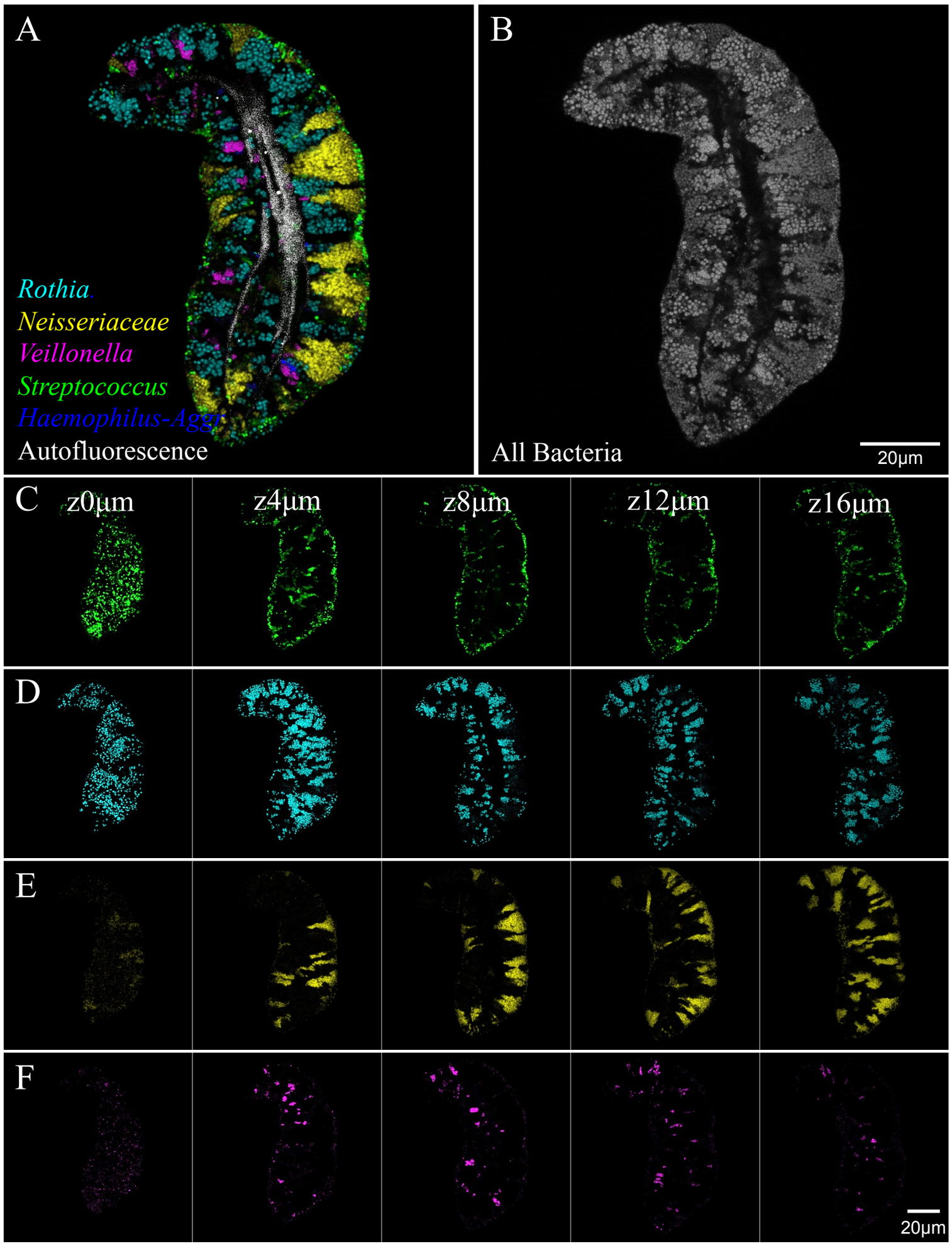
Supplemental Figure 2. **Pan-cytokeratin staining indicates that a consortium is structured around a core of keratinized epithelial cells. Related to Figure 5, Figure 6.** (A) A consortium viewed with autofluorescence (left, white) and with a pan-cytokeratin primary antibody visualized using a secondary antibody conjugated to Alexa 647 (center, red). An overlay of the two images (right) shows that the core contains cytokeratin, indicating keratinized eukaryotic epithelium. (B) Corresponding images of another consortium incubated with the secondary antibody but not the pan-cytokeratin primary antibody as a control for secondary antibody specificity.



Supplemental Figure 3. **Validation matrix demonstrating efficacy and specificity of genus and family-level probes. Related to Figure 2, Figure 3, Figure 5.** Sixteen fluorophore-labeled oligonucleotide probes were mixed in sets of 8 and applied in fluorescence *in situ* hybridization to 16 pure cultures of bacterial cells. Cultures were imaged under identical conditions and the images were subjected to linear unmixing. Each row shows a different bacterial culture and each column shows the unmixed image corresponding to a different probe. Display intensity in each column is normalized to the brightest intensity in that column. Strong signals along the diagonal of the matrix indicate hybridization of each probe to its intended target cells. Most probes show negligible hybridization to non-target cells. Exceptions, e.g. *Oribacterium* cells reacting with the *Actinomyces* genus probe Act118, create ambiguity in taxon identification that can be mitigated by the use of nested probes with overlapping specificity (Fig. S2). For example, the *Actinomyces* cells in Fig. 4 B,C are unambiguously identified by the combination of a genus and species level probe.

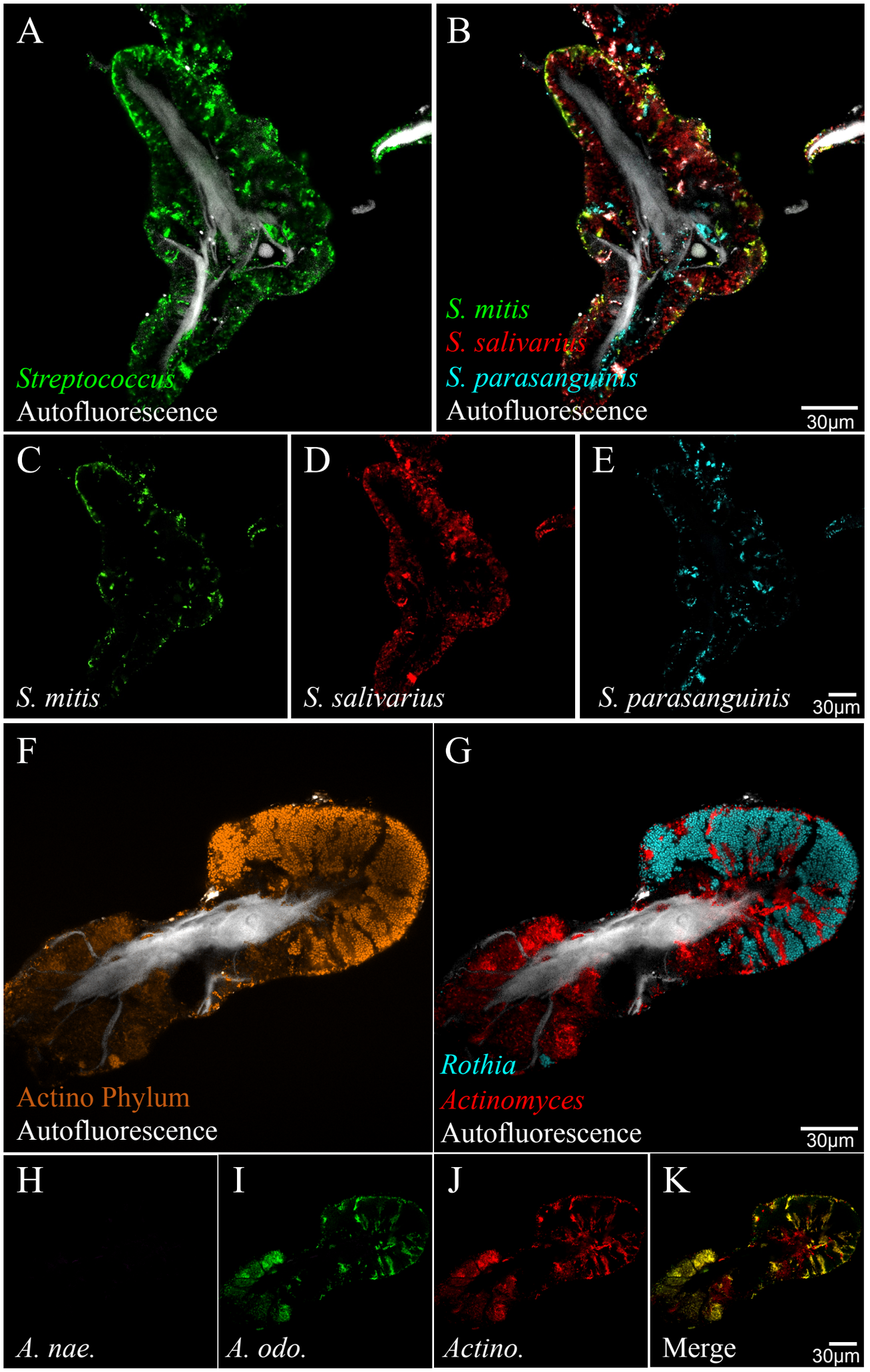


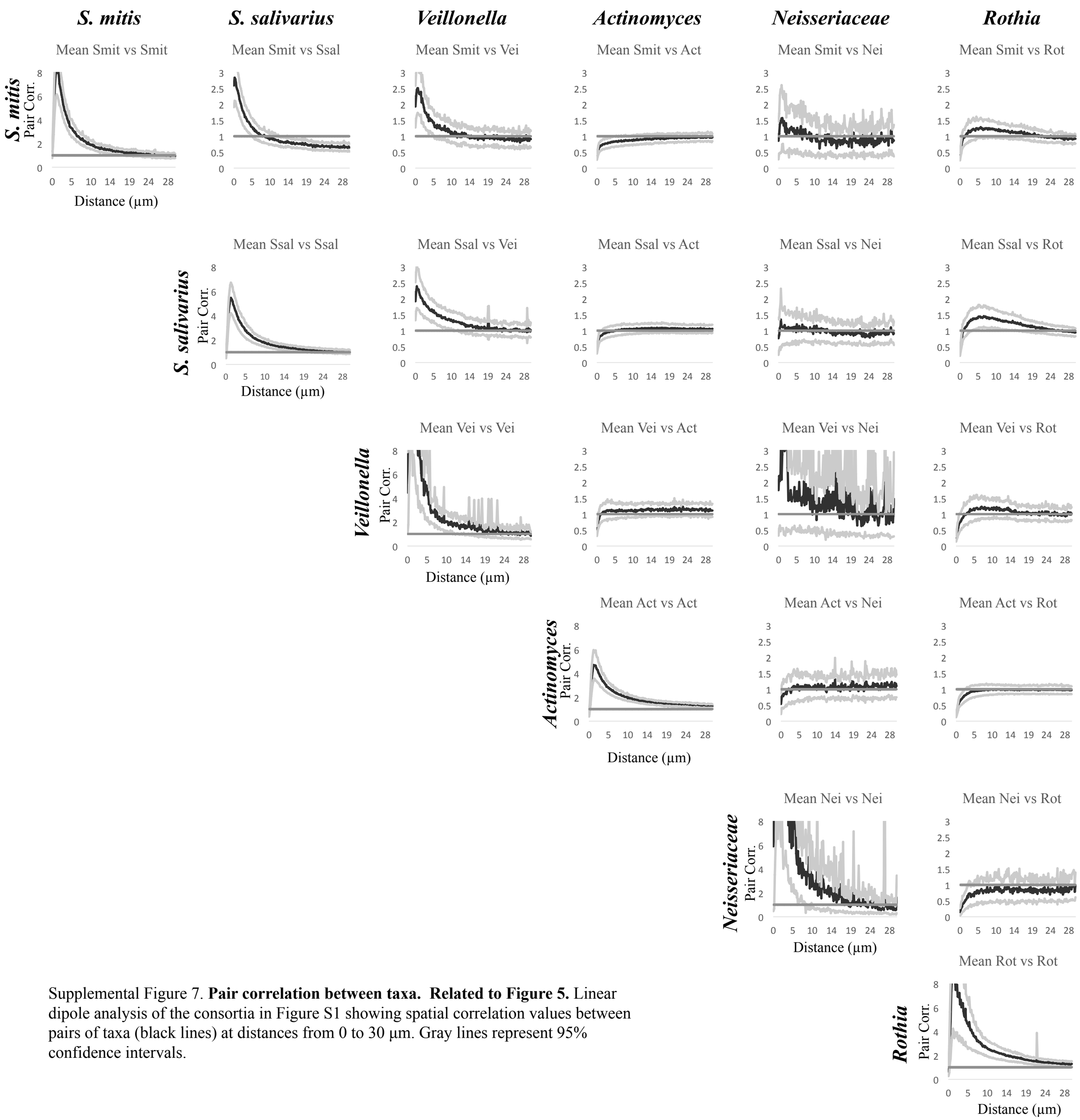
Supplemental Figure 4. **Validation matrices demonstrating efficacy and specificity of species-level probes. Related to Figure 4.** Probes targeting abundant tongue-specialist species from the genera *Rothia*, *Actinomyces*, *Neisseria*, and *Streptococcus*, as well as phylum Saccharibacteria (TM7), were tested on cultivated representatives of both target and non-target (dental plaque-abundant) species in these genera. (A-C) The universal probe Eub338 was mixed with a species-specific probe, as shown, and applied to pure cultures of on- and off-target species, hybridized under identical conditions and imaged and displayed with identical settings. (B) and (C) each show two separate hybridizations; the Dy490 (species probe) channel is shown for each and the RRX (Eub338) panel for the target taxon is shown. (D) The Str405 genus probe was mixed with a *Streptococcus* species group probe and applied to both on- and off-target *Streptococcus* species. The Dy490 (species probe) channel is shown along with the RRX (genus probe) panel for the on-target reaction. (E) The Act118 (*Actinomyces* genus) probe was mixed with the TM7550 (TM7) probe and applied to a co-culture of *Actinomyces* with Saccharibacteria (TM7) and to a pure culture of *Actinomyces*.



Supplemental Figure 5. **Three-dimensional organization of a consortium. Related to Figure 5, Figure 6.** For this representative consortium, the full-color image (A) is an overlay of five genus- or family-level probes plus autofluorescence, showing the relationship of the taxa to one another and to the epithelial core at this medial plane. The grayscale image (B) shows fluorescence at that same plane from the Eub338 probe, showing that the 5 highlighted taxa represent the majority of the Eub338-positive cells in the image. Separate fluorophore channels shown at 5 different focal planes in the same object (C-F) show the localization of *Streptococcus* around the exterior of the object, of *Rothia* and *Neisseriaceae* in wedge-shaped domains, and of *Veillonella* in interior domains. Planes in (C-F) are 0-16um deep at 4um intervals. See Supplemental Video 1 for a flythrough of the entire 20-micron z-stack at an 0.5-micron step size.

Supplemental Figure 6. **Localization of species. Related to Figure 4.** (A)-(E): *Streptococcus* species show differential localization within consortia. A nested probe set subdivides cells of the *Streptococcus* genus into three distinct species groups with distinct localization patterns. (A) Genus probe (Str405) and autofluorescence showing location of the epithelial core. (B): overlay of probes for *S. mitis* (green), *S. salivarius* (red), and *S. parasanguinis* (cyan). *S. mitis* is generally localized to the perimeter of the consortium; *S. parasanguinis* occupies small interior patches; and *S. salivarius* forms larger patches spanning from center to perimeter. Individual species channels are shown separately in panels (C), (D), and (E). Scale bar equals 30 microns. **(**F)-(K): Nested probe set provides three-level identification of *Actinomyces* cells within consortia. (F) A nested probe set containing probes with distinct fluorophores for the phylum Actinobacteria, the genera *Actinomyces* and *Rothia*, and species groups for *A. odontolyticus* and for *A. naeslundii* was applied to donor material in order to assess and confirm identification of *Actinomyces* cells. Cells hybridizing with the Act382 phylum probe are shown in panel (F) together with autofluorescence showing location of the epithelial core. Genus probes for *Rothia* (Rot491) and *Actinomyces* (Act118) together identify nearly all cells identified with the Act382 phylum probe (G). *A. odontolyticus* species group accounts for majority, but not all, of *Actinomyces* cells in this consortium (compare I with J; overlay shown in K). No cells appeared labeled by the probe targeting *A. naeslundii* (Act476, panel (H)), corroborating sequence analysis results. Scale bar equals 30 microns.





Supplemental Figure 7. **Pair correlation between taxa. Related to Figure 5.** Linear dipole analysis of the consortia in Figure S1 showing spatial correlation values between pairs of taxa (black lines) at distances from 0 to 30 µm. Gray lines represent 95% confidence intervals.

Probe Name	Target Taxon				Probe Sequence 5'-3'	Reference
	Domain	**Family**	**Genus**	**Species**		
Eub338	Bacteria				GCTGCCTCCCGTAGGAGT	Amann et al. 1990
Actinobacteria						
Act118			*Actinomyces*		GGCAGGTTACTCACGTGTT	This Paper
Rot491			*Rothia*		TAGCCGGCGCTTTCTCTG	Valm et al. 2011
Aodo475				*A.odontolyticus* group	TTACCCACTACCCTCACCA	This Paper
Agra475				*A.graevenitzii*	CTTATCCAGGTACCCTCAACAC	This Paper
Rmuc435				*R.mucilaginosa*	TCTCTTCTTCCCTGCTAACAG	This Paper
Cor430		Coriobacteriaceae	*Atopobium, Olsenella*		TCCCTGCTGAAAGCGGTT	This Paper
Bacteroidetes						
Pre392			*Prevotella, Alloprevotella*		GCACGCTACTTGGCTGG	Diaz et al. 2006
Por1160			*Porphyromonas* (subset)		CCTCACGCCTTACGACGG	Valm et al. 2011
Cap371			*Capnocytophaga*		TCAGTCTTCCGACCATTG	Zijnge et al. 2010
Firmicutes						
Str405			*Streptococcus*		TAGCCGTCCCTTTCTGGT	Paster et al. 1998
Vei488			*Veillonella*		CCGTGGCTTTCTATTCCG	Chalmers et al. 2008
Gem572			*Gemella*		TAAACCACCTGCGCGCGCTT	Valm et al. 2011
Smit651				*S.mitis* group	CCCCTCTTGCACTCAA	This Paper
Ssal372				*S.salivarius* group	AGGGTTGCCCCCATT	This Paper
Lac432		*Lachnospiraceae*			TCTTCCCTGCTGATAGAGCT	This Paper; Kong et al. 2010
Gra65			*Granulicatella*		GCACCGGTCGCTCTCGTT	Valm, This Paper
Proteobacteria						
Nei1030		*Neisseriaceae*			CCTGTGTTACGGCTCCCG	Valm et al. 2011
Pas111		*Pasteurellaceae*	*Haemophilus, Aggregatibacter*		TCCCAAGCATTACTCACC	Valm et al. 2011
Cam1021			*Campylobacter*		ATTTCTGCAAGCAGACACTC	Valm et al. 2011
Nsubfla177				*N. flavescens* group, *N.subflava*	CTTTCCTCCTCAGAGAATATG	This Paper
Nfla469				*N.flavescens* group	GTACCGTCATCAGCTGTCG	This Paper
Fusobacteria						
Fus714			*Fusobacterium*		GGCTTCCCCATCGGCATT	Valm et al. 2011
Lep568			*Leptotrichia*		GCCTAGATGCCCTTTATG	Valm et al. 2011
TM7						
TM7550		*TM7 (Saccharibacteria)*			CCCAGTCACTCCGGATAA	Valm, This Paper

Supplemental Table 2. **Probes employed in this study. Related to Figure 2, Figure 3, Figure 4, Figure 5, Figure 6.** Probes are listed by phylum; the probe name, target taxon, and probe sequence are shown.

Oligo-nucleotide Probe Name	Probe Set 1	Probe Set 2	Probe Set 3	Probe Set 4	Probe Set 5	Probe Set 6	Probe Set 7	Probe Set 8	Probe Set 9	Probe Set 10	Probe Set 11	Probe Set 12	Probe Set 13	Probe Set 14	Probe Set 15
Eub338				Dy 415 Dual	Dy 415 Dual	Dy 415 Dual	Dy 415 Dual	Dy 415 Dual				Dy 510XL Dual			Dy 415 Dual
Rot491	Atto 655 Dual	Atto 655 Dual	Atto 655 Dual	Atto 655 Dual	Atto 655 Dual	Atto 655 Dual	Atto 655 Dual	Atto 655 Dual	Atto 655 Dual	Atto 655 Dual	Atto 655 Dual	Atto 655 Dual	Atto 655 Dual	Atto 655 Dual	Atto 655 Dual
Str405		Atto 532 Dual	Atto 532 Dual	Atto 532 Dual	Atto 532 Dual	Atto 532 Dual	Atto 532 Dual	Atto 532 Dual	Atto 532 Dual	Atto 532 Dual		Alexa 488	Rhodamin Red X Dual		Atto 532 Dual
Vei488	Atto 550	Atto 550	Atto 550	Atto 550	Atto 550	Atto 550	Atto 550	Atto 550	Atto 550	Atto 550	Atto 550	Alexa 514			Atto 550
Act118	Texas Red X	Texas Red X	Texas Red X	Texas Red X	Texas Red X	Texas Red X	Texas Red X	Texas Red X	Texas Red X	Texas Red X	Texas Red X			Texas Red X	Texas Red X
Fus714		Pacific Blue Dual	Dy 415 Dual						Dy 415 Dual						Alexa 514
Pre392	Atto 520 Dual		Alexa 488								Dy 505 Dual				Alexa 488
Lep568		Atto 647n	Rhodamin Red X						Rhodamin Red X						Rhodamin Red X
Nei1030	Atto 620 Dual	Atto 620	Atto 633 Dual	Atto 633 Dual	Atto 633 Dual	Atto 633 Dual	Atto 633 Dual	Atto 633 Dual	Atto 633 Dual	Atto 633 Dual	Atto 620	Atto 633 Dual			Atto 633 Dual
Gra65			Alexa 514												
Pas111		Atto 594 Dual	Dy 615 Dual									Dy 615			
Por1160		Atto 425 Dual										Dy 485XL			
TM7550		Rhodamin Red X									Rhodamin Red X Dual				
Gem572									Alexa 488						
Lac432												Texas Red X			
Smit651	Dy 415 Dual										Dy 415 Dual		Dy 415 Dual		
Ssal372	Dy 490 Dual										Dy 490 Dual		Dy 490 Dual		
Spar21													Rhodamin Red X Dual		
Rmuc435				Dy 490											
Nsubfla177								Dy 490							
Nfla469							Dy 490								
Aodo475					Dy 490									Dy 490	
Agra475						Dy 490									
Act382														Dy 415 Dual	
Act476														Dy 510XL Dual	
Shown in:	Fig. 2, Fig. 4F, Fig. 6D, Fig S1, Vid. 3	Fig. 3A	Fig. 3B	Fig. 4A	Fig. 4B	Fig. 4C, Fig. 6E	Fig. 4D	Fig. 4E	Fig. 5	Fig. 6 A-C	Fig. 6F	Fig. S5, Vid. 1	Fig. S6 A-E	Fig. S6 F-K	Vid. 2

Supplemental Table 3. **Probe sets employed in figures. Related to Figure 2, Figure 3, Figure 4, Figure 5, Figure 6.** For each set, the table lists the probes used (oligonucleotide and fluorophore) as well as the figure panels in which that probe set is shown. "Dual" indicates that the probe was labeled with the same fluorophore at both 5' and 3' ends; if "dual" is not specified, the probe was labeled only at the 5' end.

Genus Level Imaging-Based Prevalence					
Target	**Probe**	**Subjects**	**Prevalence (%)**	**Images**	**Frequency (%)**
Actinomyces	Act118	11	100	152	98
Streptococcus	Str405	20	100	325	96.6
Rothia	Rot491	20	100	365	94.8
Veillonella	Vei488	20	100	346	85.3
Gemella	Gem572	12	100	32	75
Neisseriaceae	Nei1030	20	100	284	69
Saccharibacteria (TM7)	TM7550	6	100	56	32.1
Granulicatella	Gra65	20	95	128	80.5
Prevotella	Pre392	12	91.7	145	52.4
Fusobacteria	Fus714	19	89.5	173	60.1
Capnocytophaga	Cap371	8	62.5	37	16.2
Leptotrichia	Lep568	13	53.8	85	40
Porphyromonas	Por1160	16	50	56	23.2
Haemophilus-Aggregatib.	Pas111	20	40	130	16.2
Atopobium	Cor430	16	37.5	63	28.6
Campylobacter	Cam1021	17	17.6	104	2.9
Lachnospiraceae	Lac432	14	7.1	44	43.2
Corynebacterium	Cor633	6	0	23	0

Species Level Imaging-Based Prevalence					
Target	**Probe**	**Subjects**	**Prevalence (%)**	**Images**	**Frequency (%)**
A. odontolyticus	Aodo475	10	100.0	30	100.0
R. mucilaginosa	Rmuc435	10	100.0	10	100.0
S. mitis	Smit651	4	100.0	40	100.0
S. salivarius	Ssal372	4	100.0	40	95.0
N. subflava +N. flavescens	Nsubfla177	10	100.0	11	90.9
N. flavescens	Nfla469	10	70.0	10	70.0
A. graevenitzii	Agra475	10	30.0	10	30.0

Supplemental Table 4. **Prevalence and abundance of taxa in tongue consortia assessed by imaging. Related to Figure 3.** Twenty-five FISH probes were used to assess the prevalence and abundance of taxa in tongue consortia from up to 20 subjects. Seventeen probes targeted genus- or family-level taxa, 7 targeted species-level groups, and one targeted the phylum Saccharibacteria (TM7). Probes were employed in a variety of probe-set combinations and the number of images in which a taxon was detected was tallied for at least 10 and as many as 365 images. Prevalence was calculated as the percent of tested subjects in which the taxon was detected; frequency was calculated as the percent of images in which the taxon was detected.